Functional and Genetic Interactions of Ribosomal-like Protein 24

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Abstract

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Nearly all the processes within a cell are mediated by proteins: metabolism, movement, growth, and even death. These proteins are generated by a type of intracellular machinery called the ribosome which translates the cell's genetic code into a functional protein. But where and how are ribosomes generated? Current research indicates that ribosomes are first made into two separate parts near the genetic material stored in the cell's nucleus. The new subunits are passed through a discriminating gateway called the Nuclear Pore Complex (NPC) that separates the nucleus from the remainder of the cell's interior, or cytoplasm. The ribosomes are very large, however, and cannot pass through the NPC on their own. It has been found that some proteins function to "chaperone" the ribosomal subunits out of the nucleus by assisting interaction between the ribosome and the NPC. We suspect a certain protein in yeast, Ribosomal-like Protein 24 (Rlp24), is involved in this export pathway. Its specific function is not yet known, but it has been previously determined that it binds to the ribosome in the nucleus and then is unloaded in the cytoplasm.

In order to determine if this protein has a role in ribosome export, several genetic and functional tests were performed. In the first experiment, an altered form of Rlp24 with a molecular tag was expressed in cells that are deficient in specific proteins that comprise the Nuclear Pore Complex. A change in growth with these mutations suggests a functional interaction between the two proteins. Next, a screen for a gene that would restore normal growth in these mutants was done, suggesting that any of the potential genes also interact in the

pathway. Finally, a special screen called a Yeast Two-Hybrid assay was used to identify proteins that actually bind to and function with Rlp24.

From this series of tests, it was determined that Rlp24 does in fact interact functionally with specific components of the Nuclear Pore Complex, several of which have previously been identified as interacting in known export pathways. Additionally, the other screens identified one novel genetic interaction as well as two functional interactors with Rlp24. Though one physical interactor, a protein called Nog1, has already been cited in literature, the role of the other interactors in export and their relationships with Rlp24 are not yet known. However, the information gathered highly suggests that Rlp24 has a role in export and provides a basis for further testing and characterization.

Contents

Background

The importance of ribosomes for a cell's growth and survival is unquestionable. Ribosomal defects are seen in such human ailments as Schwachman-Bodian-Diamond syndrome or Diamond Blackfan anemia, both of which produce predominantly childhood complications and abnormalities such as loss of pancreatic function or anemia (Menne, Goyenechea et al. 2007; Choesmel, Fribourg et al. 2008). Studies in other vertebrate organisms such as mice and zebrafish show significant problems when component ribosomal proteins are altered or missing. This includes defects in organ development and even some cancers (Panic, Tamarut et al. 2006; Uechi, Nakajima et al. 2006). The real importance of the ribosome is seen in antibiotics we take to rid ourselves of bacterial infections like tuberculosis or *Pseudomonas aeruginosa*, one of the most common infections acquired in hospital stays. Antibiotics such as streptomycin affect the function of the bacterial ribosome, inhibiting bacterial protein production selectively and effectively killing the intruders. The necessity for ribosomes is clear but the mechanisms of its synthesis and assembly are not. An introduction to these processes is essential in understanding the importance of this study.

The Nuclear Pore Complex

In eukaryotes, the nucleus serves as a protective chamber for the genetic information stored in DNA as well as a compartment for RNA processing and editing. However, molecules, proteins, and other materials must pass in and out of the nucleus to access this information and allow many of the cell's essential processes to occur. The Nuclear Pore Complex (NPC) acts as a discriminating gateway between the nucleus and the rest of the cell's interior, the cytoplasm, which strictly regulates both import and export of all large material. Yet, a large quantity of material must quickly and efficiently travel between the nucleus and the cytoplasm. In humans,

for example, it is estimated that up to one kilogram of material is passed through all of the body's Nuclear Pore Complexes each minute (Weis 2007). The NPC, therefore, must maintain a certain amount of selectivity in transport as well as speed and efficiency.

The NPC is a large protein complex containing approximately 30 distinct proteins, called nucleoporins, each present in multiple copies (Rout and Aitchison 2001). The complex has eight-fold symmetry and three basic domains. The inner and outer domains are similar in that both the cytoplasmic and nuclear faces of the NPC contain filaments that project away from the pore. Unlike the cytoplasmic face the filaments on the nuclear face join together to create what is termed the nuclear basket (Rout, Aitchison et al. 2000). The center of the pore contains a cylindrical core with eight radiating spokes, creating an opening approximately 38 nanometers in width (Alber, Dokudovskaya et al. 2007). Not surprisingly, this limits this size of proteins and large molecular complexes that may pass in and out of the nucleus. Some small molecules (less than 40 kDa) can pass through the NPC unassisted. However, larger proteins require either a direct interaction with the proteins of the NPC or a transport receptor that facilitates movement through the pore (Tran and Wente 2006).

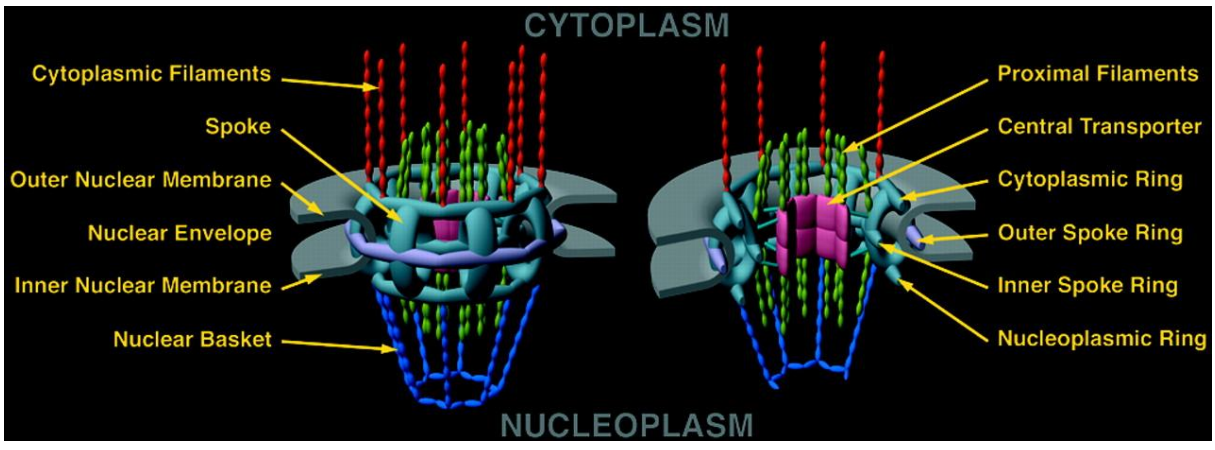

Figure 1 Proposed structure of the Nuclear Pore Complex. The three main domains are clearly identified: cytoplasmic filaments in red, nuclear basket in blue, and central channel composed of spoke rings embedded in the membrane. Reprinted by permission (Rout and Aitchison 2007).

The structure and composition of the interior space of the pore is a current area of research (reviewed in Weis 2007). Many of the nucleoporins in the channel contain chains of the amino acids phenylalanine (F) and glycine (G) (Tran and Wente 2006). These chains are hydrophobic, or water repelling. One idea is that these chains project into the pore, blocking the flow of particles (**Figure 2A**). Proteins that bind these FG repeats, as they are called, can help to push the projections out of the way, allowing passage of molecules. Another model asserts that these chains not only project into the pore, but that they interact with each other in random associations to create a meshwork that acts like a sieve (Ribbeck and Gorlich 2001). This would "filter" out molecules too large to pass into the nucleus (**Figure 2B**). As an extension to the previous model, it has also been suggested that the meshwork of FG repeats is not random but highly ordered (**Figure 2C**), creating a gel-like selectively permeable barrier (Frey, Richter et al. 2006). The strict regulation of transport by the Nuclear Pore Complex appears to be a result of the number and composition of the nucleoporins.

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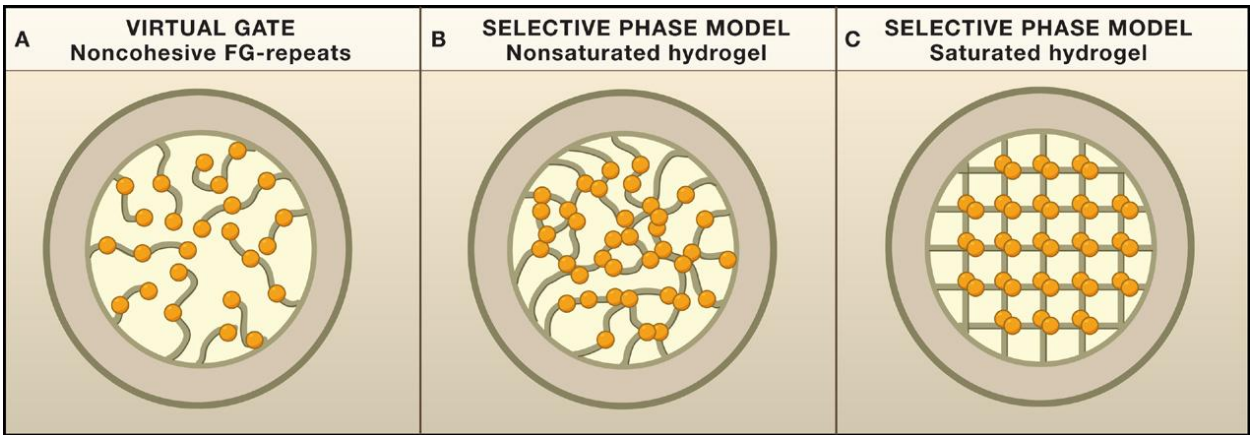


Figure 2 Proposed models of the interior of the NPC. Non-associative FG projections are seen in 2A, while 2B and C show levels of increasing association and organization of FG repeats. The composition of this internal channel could be partially responsible for the selectivity of the NPC. Reprinted by permission, (Weis 2007).

Ribosome Biogenesis and Function

Almost every function in the cell is mediated by proteins. The ribosome is the cell's machinery that is able to read genetic messages and turn them into proteins. Ribosomes themselves are large groupings, or complexes, of proteins and a type of genetic material called RNA. Ribosomes are composed of two subunits: a large subunit called 60S and a small subunit called 40S. After synthesis of their constitutive parts (4 rRNAs and over 80 proteins), these proteins and RNAs are assembled into a large complex that eventually breaks into the developing 40S and 60S subunits in a small region of the nucleus called the nucleolus (Zemp and Kutay 2007). Each subunit must then make its way to the cytoplasm via the nuclear pore complex.

Concurrently, message copies of certain genes in DNA called mRNA also pass through the NPC. Once in the cytoplasm, the 40S and 60S subunits associate with the mRNA and begin to "read" the gene. The ribosome helps to assemble specific building blocks called amino acids to create a protein that the gene's mRNA encodes. In fact, many ribosomes can associate successively with the same mRNA, creating many copies of the encoded protein quickly and efficiently. Once finished, the subunits dissociate from the mRNA and are free to bind to new mRNA to make more protein. This process is called translation; the genetic code is translated into amino acids, or protein (Nelson and Cox 2005).

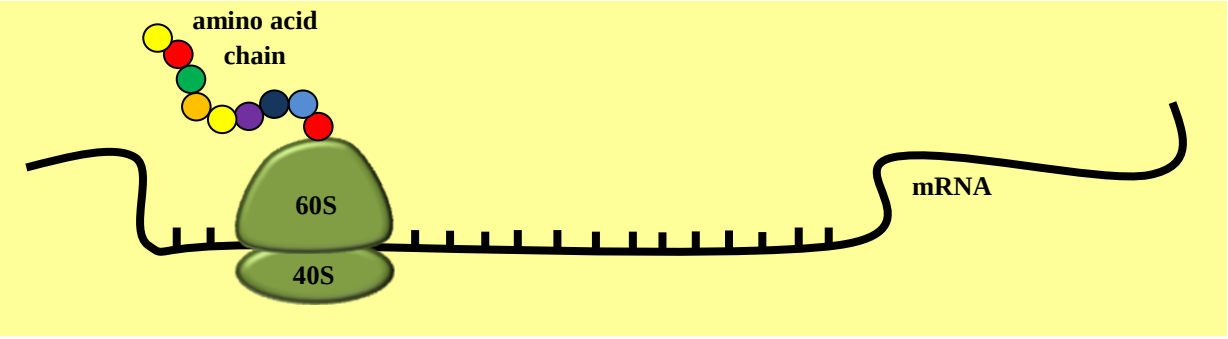


Figure 3: Cartoon diagram of translation. The ribosomal subunits situate themselves around a strand of mRNA at a particular nucleotide sequence of ATG. The ribosome "reads" the genetic code as it travels 5' to 3' and generates a strand of amino acids corresponding to the code.

Ribosomal Export

The ribosomal subunits are extremely large complexes measuring about 23 nanometers in diameter, reaching the upper limits of the span of the nuclear pore (Nelson and Cox 2005). Additionally, the RNA component of each contains an extremely negative charge. This tends to prevent association with the hydrophobic projections in the center of the NPC, like the interaction between oil and water. Obviously, the ribosomes must overcome the size and charge barriers to passing into the cytoplasm.

Most large macromolecules destined for import or export must contain certain "signals" that the transport machinery can recognize to direct them either in or out. These generally come in two varieties, Nuclear Localization Signals (NLS) that facilitate import into the nucleus and Nuclear Export Signals (NES) that help get the molecule from the nucleus to the cytoplasm. These signals are recognized and bound by specific molecules called karyopherins, which assist in moving the cargo through the pore. These molecules use energy in the form of the molecule GTP to assist the import or export of cargoes (Kutay and Guttinger 2005). In ribosomal export, a karyopherin called Crm1 is one of the major molecules responsible for export. It binds to the NES sequence on a protein called Nmd3, which binds directly to the large ribosomal subunit (Ho, Kallstrom et al. 2000). This interaction is needed for the large subunit to be efficiently exported. However, with such a large molecule an increased number of these transport receptors may enhance efficiency and expedience in export (Ribbeck and Gorlich 2001).

Furthermore, with the theory that the interior of the pore complex is comprised of a hydrophobic sieve, it is likely that some proteins must bind to the hydrophobic regions to open a passage for molecules to travel. One such presumptive mechanism is export by the receptor complex Mtr2 and Mex67. These two receptors were previously known to assist in export of mRNA from the nucleus, but in budding yeast a binding site for them was found on the 60S

subunit as well, suggesting a role in export (Yao, Roser et al. 2007). Mtr2-Mex67 can bind to the FG repeat hydrophobic region of the nucleoporins while simultaneously interacting with the 60S subunit on another face. Additionally, the binding site of Mtr2-Mex67 is distinct from the binding sites for Nmd3 and another export receptor Arx1, suggesting the potential redundancy of multiple export receptors (Yao, Roser et al. 2007). Arx1 is a protein most recently attributed to assisting 60S ribosomal export. Interactions with nucleoporins in the NPC as well as impaired export in its absence suggest its role in export (Bradatsch, Katahira et al. 2007; Hung, Lo et al. 2008). Its mechanism is thought to be similar to that of Mtr2-Mex67 in that it associates to the hydrophobic regions in the NPC core. It is hypothesized that all pathways, Nmd3/Crm1, Mtr2-Mex67, and Arx1, are used together rather than as separate export mechanisms. Tandem pathways could be necessary for efficient export by avoiding competition between receptors, or different binding sites for each of the receptors could help orient the large 60S subunit with respect to the NPC so that it may physically fit through the pore (Hung, Lo et al. 2008). The multiplicity of these pathways encourages the idea that more pathways and more receptors could exist. Additional proteins may also be involved in the known pathways but are not yet characterized.

Ribosomal-like Protein 24

RLP24 is an essential gene in yeast, meaning its removal causes cell death. Like most essential proteins, it is conserved between many eukaryotes and even has a human homolog. The Rlp24 protein is comprised of 199 amino acids and is approximately 29 kDa in size. Its name comes from the homology it bears to Rpl24 (Ribosomal Protein of the Large Subunit 24), which binds to the 60S subunit in the cytoplasm and assists the ribosome in efficient translation. It is suggested that the homology allows Rpl24 to replace and occupy the same binding site of

Rlp24 on the ribosome. Essentially, Rlp24 is transiently involved in 60S subunit biogenesis and export from the nucleus and then is replaced by a protein involved in efficient translation in the cytoplasm (Saveanu, Bienvenu et al. 2001). Interestingly, Rlp24 is essential whereas Rpl24 is not.

Rlp24 was first implicated in having a role in ribosome biogenesis because of its interaction with another protein determined to be essential for the process, Nop7p. As mentioned before, strains lacking Rlp24 cannot survive, and when expression of the gene is "shut off" in cells it results in a decrease of 60S subunits, suggesting its function in proper formation of subunits (Harnpicharnchai, Jakovljevic et al. 2001). Additionally, the cellular localization of Rlp24 was examined to determine where and when Rlp24 might interact with the ribosome biogenesis pathway. Rlp24 protein was found in both the nucleus and cytoplasm, with a slight preference to the nucleolus (Saveanu, Namane et al. 2003). Further analysis showed that all of the cytoplasmic Rlp24 was bound to the large ribosomal subunit, and in strains with deficient 60S nuclear export, Rlp24 is localized only to the nucleus. This suggests that Rlp24 helps in maturation of the subunit in the nucleus and shuttles to the cytoplasm during export (Saveanu, Namane et al. 2003). Therefore, Rlp24 has an essential role in ribosome biogenesis and an implied function in export based on its localization patterns.

Several of the interactions between Rlp24 and other proteins have been examined in the context of ribosome maturation and biogenesis in the nucleus. The goal of this study, therefore, is to begin to test genetic interactions that may imply a role of Rlp24 in export. This may provide insight into the pathways in which Rlp24 acts or similarities between Rlp24 and known export receptor mechanisms. It may also reveal novel interactions not previously known to be involved in ribosomal export.

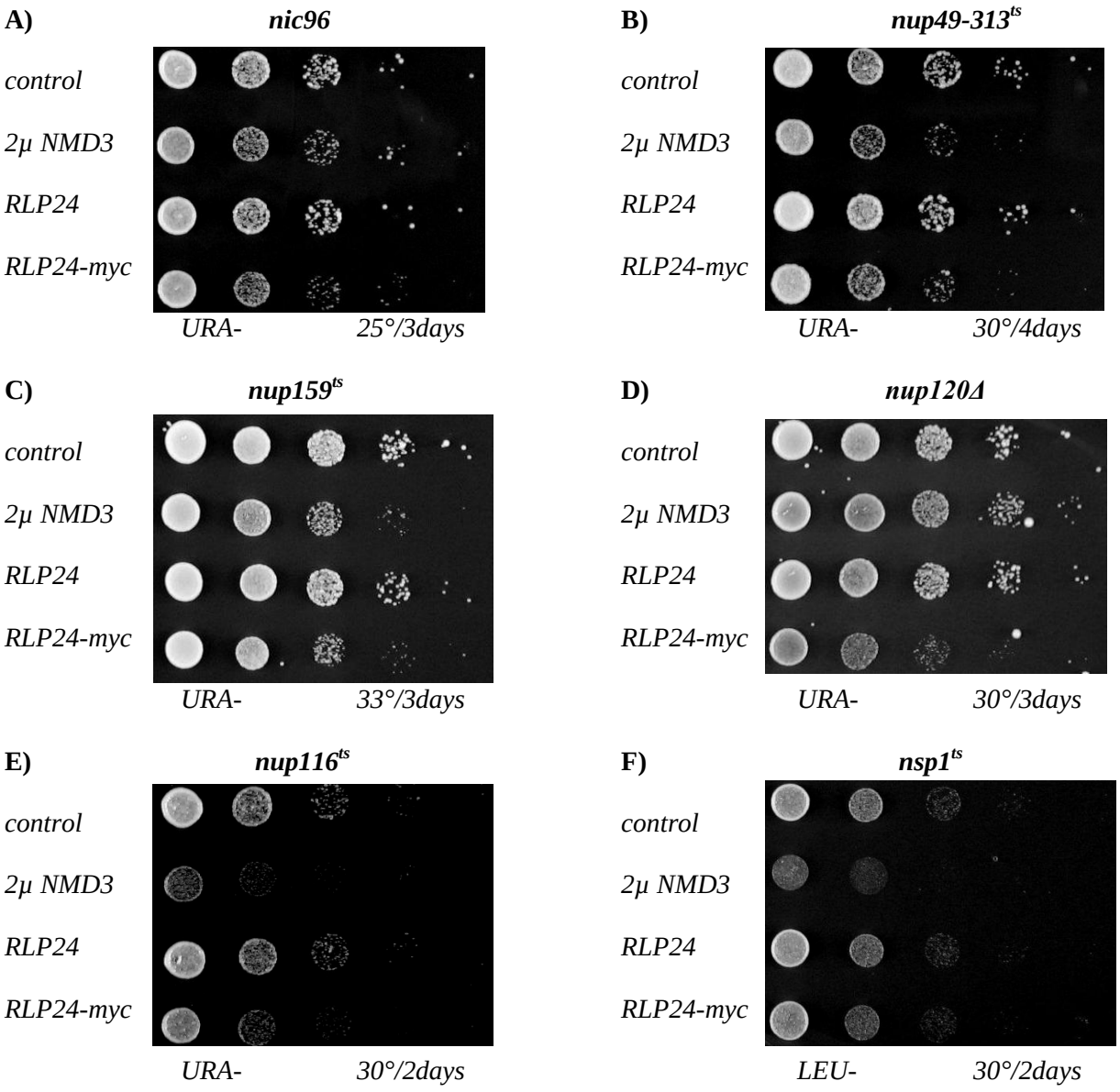
Results

Dominant Negative Expression of RLP24-myc in nup Deficient Strains

In the first phase of this project, the genetic relationship between Rlp24 and several nucleoporins was investigated. Both *RLP24* and *RLP24* with a 13 myc epitope tag were expressed in yeast strains deficient for specific nucleoporins. Prior to this study, it was observed in our hands that *RLP24*-myc showed a dominant negative phenotype, a phenomenon also seen when over-expressing *NMD3* or *CRM1*. Over-expression of Nmd3 is thought to occupy binding sites on Crm1, preventing it from interacting with Nmd3 bound to the ribosome and thus hindering export. This led us to question whether the effect seen with *RLP24*-myc occurs by a similar mechanism. Therefore, a vector over-expressing *NMD3* was also transformed into these same strains. An empty vector containing the same selective marker as these plasmids was used as a negative control. Cell cultures of the transformants were diluted to the same optical density to control plating of equivalent numbers of cells. Five microliters of a 10x serial dilution from 10^{0} to 10^{-4} was plated for each transformant on appropriate selective media. Plates were made in triplicate and incubated at 25°, 30°, and 33°.

In all strains, the expression of untagged *RLP24* did not result in a change in phenotype or growth rate as compared to the control. *RLP24*-myc, however, showed a significant dominant negative phenotype in certain strains. The strength of the phenotype varied, especially in strains with temperature sensitive nup mutations. In all strains with the *RLP24*-myc dominant negative phenotype, overexpression of *NMD3* also produced a dominant negative phenotype. A complete chart of all phenotypes is included in **Table 4** in Appendix 1 while representative images for select strains are shown in **Figure 4** below.

Figure 4: Dominant Negative Phenotype with Expression of *RLP24*-myc and High Copy *NMD3* in nup Mutant Strains



Figures show the expression of *RLP24*-myc and *NMD3* compared to controls. *RLP24* without the myc tag shows no growth defect in any strain as compared to control (URA or LEU empty vector). Growth is shown at indicated times and temperatures which showed maximum phenotype. 4A) shows expression in the *nic96* strain at room temperature, showing a slight dominant negative effect (D/N). Expression at 30° and 33° resembles control (not shown). 4B) shows strong D/N in both *NMD3* and *RLP24*-myc at all temperatures in *nup49*^{ts} mutant strain. *Nup159*^{ts} mutant (4C) shows a strong D/N phenotype at 33°, but slightly less at RT and 30° (not shown). *Nup120Δ* (4D) has only a slight D/N with *NMD3* but an extremely strong phenotype for *RLP24*-myc. 4E) shows *nup116*^{ts} mutant and its very strong D/N effects for both *NMD3* and *RLP24*-myc while the *nsp1*^{ts} mutant (4F) shows less D/N in *RLP24*-myc but strong D/N *NMD3*.

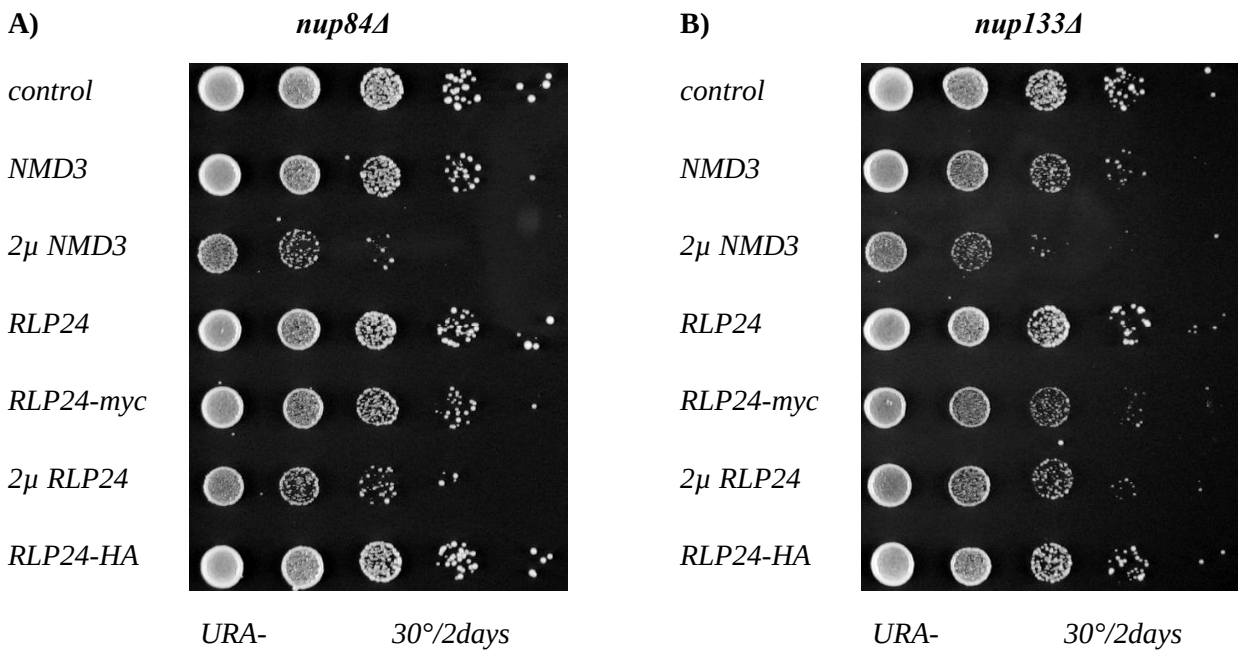
The strongest dominant negative phenotype with *RLP24*-myc was seen in the *nup120* deletion strain. The *nup159*^{ts} mutant also showed a strong phenotype, especially at the non-permissive temperature (33°). Other mutants showing a significant dominant negative phenotype were *nup116*^{ts}, *nup84*, *nup133*, *nup49*^{ts}, *nsp1*^{ts}, and *nic96* (**Figure 4**). *Nup82* was the only strain expressing a D/N phenotype with *NMD3* and not *RLP24*-myc (not shown).

Based on the strong phenotypes from the first screen, centromeric expression of *NMD3* and *RLP24*-myc was compared to high copy 2µ expression of *NMD3* and 2µ expression of *RLP24*. Only two strains were selected for this comparison, *nup84Δ* and *nup133Δ*. Additionally, a comparison between the 13myc tag and another epitope tag, HA, was tested to determine whether or not the specific tag was impairing function to create the dominant negative phenotype. Results showed that 2µ expression produced a stronger dominant negative phenotype for *NMD3* than CEN, while 2µ *RLP24* expression showed D/N effects equivalent to *RLP24*-myc. Increased numbers of Rlp24 as well as the myc epitope tag may block binding of export receptors disrupting potential interactions and causing export defects. *RLP24*-HA expression showed no phenotypic difference to control, indicating the HA tag was insufficient to block function of Rlp24 or other interactions necessary for ribosome export (**Figure 5**). In our hands it has also been shown that TAP tagged Rlp24 is affected, so this effect is not specific to the myc tag alone.

Overall, the genetic interactions observed closely paralleled those of Nmd3, further supporting the idea that the expression of Rlp24-myc has a similar effect on export. That is to say, the epitope-tagged protein Rlp24-myc interferes with export, possibly by blocking the binding of an export factor. Likely candidates for the affected factor are Nmd3, Crm1, Mex67, Mtr2, and Arx1, although a novel protein could be involved as well. To tease apart these mechanisms, a screen was undertaken to find a gene that when over-expressed suppressed the

growth defect of the Rlp24-myc mutants. The reasoning being that if Rlp24-myc impaired binding of a factor, increasing the dosage of the factor may drive more onto the subunit, overcoming the defect cause by Rlp24-myc.

Figure 5: Dominant Negative Phenotype with RLP24-myc, 2µ Expression of *RLP24* and CEN and 2µ Expression of *NMD3* in *nup84Δ* and *nup133Δ* Mutant Strains



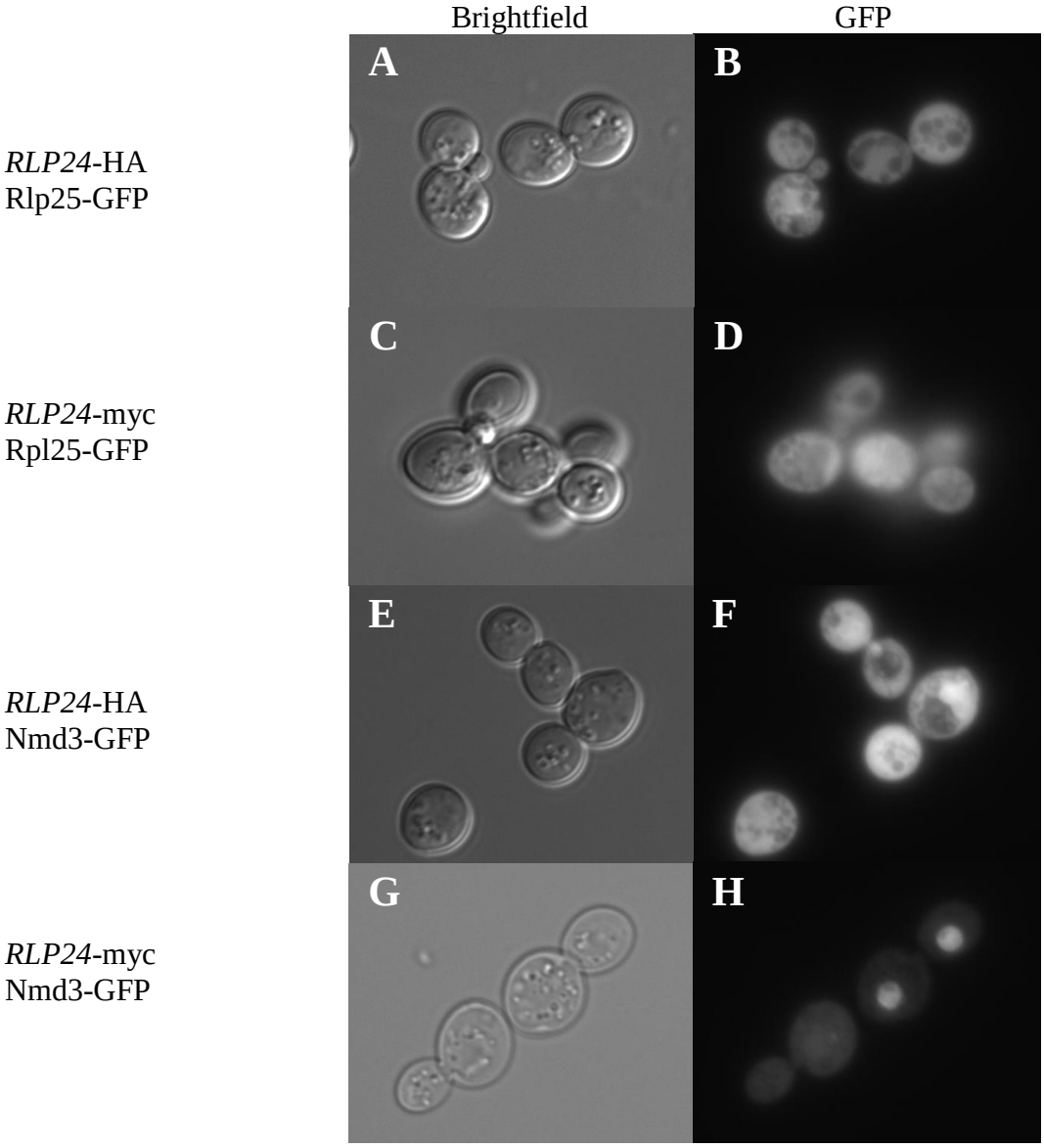
Figures 5A and B show expression of centromeric and high copy *NMD3* and high copy *RLP24*, as well as *RLP24*-HA. Both *nup84Δ* and *nup133Δ* show greatly enhanced D/N phenotype with 2µ *NMD3* as compared to CEN. D/N phenotype for 2µ *RLP24* is increased as compared to *RLP24*-myc in *nup84Δ* **(A)** and has similar growth to *RLP24*-myc in *nup133Δ* **(B)**. *RLP24*-HA has expression similar to control.

Fluorescence Microscopy of Export Defects in* RLP24-*myc Mutant Strains

To investigate whether or not export was blocked in these *RLP24*-myc mutants, Rpl25-GFP and Nmd3-GFP fluorescent export markers were transformed into strains with strong D/N phenotypes: *nup49^{ts}, nup84Δ, nup116Δ, nup120Δ, nup133Δ*, and *nup159^{ts}*. Strains with *RLP24*-HA were used as controls with uncompromised export. Cells were then examined under UV illumination to determine localization of the ribosome reporters. Most strains showed no change localization while some strains showed a slight difference with the Rpl25-GFP reporter. This is

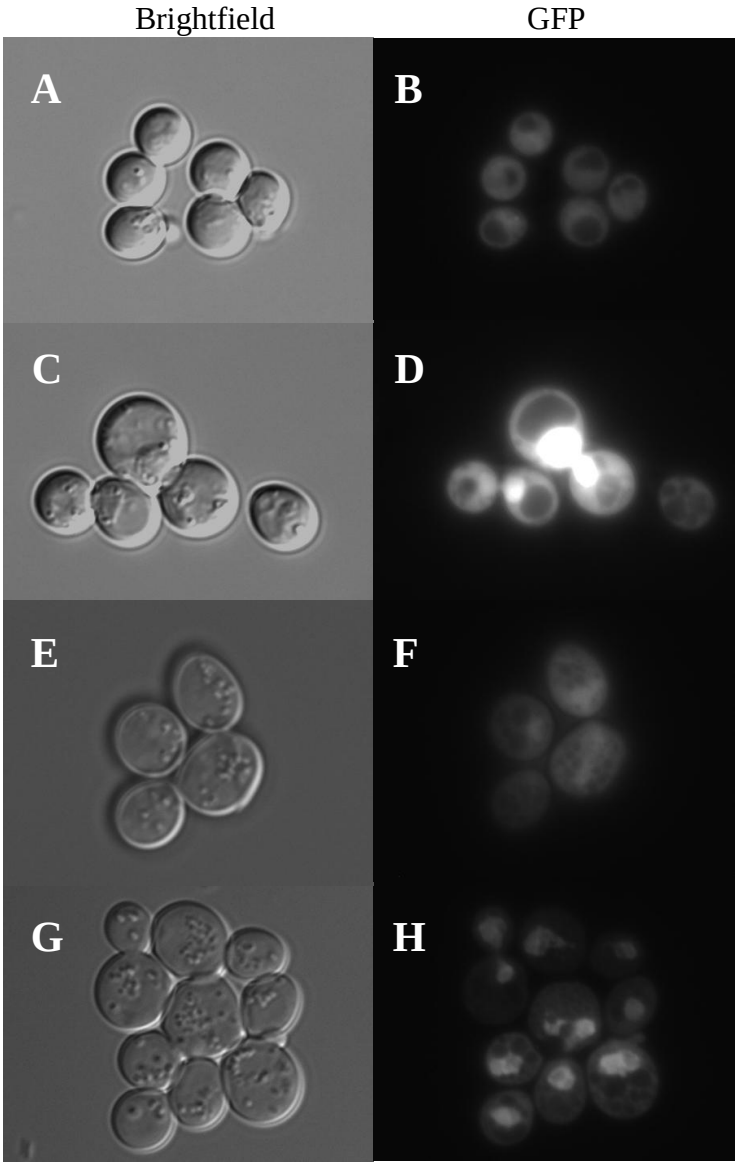
a ribosomal protein marker, so it is present on ribosomes both in the nucleus and cytoplasm. If the export defect is not strong, the localization difference may be negligible. Nmd3-GFP is a stronger reporter because it is an export receptor, so export defects prevent its shuttling to the cytoplasm (representative pictures seen in **Figures 6 & 7**).

Figure 6: *RLP24*-myc expression in *nup120Δ* causes slight export defects as observed with Nmd3-GFP



Panels A, B, E, and F show normal localization for Rpl25-GFP and Nmd3-GFP in a *nup120Δ* strain. In C and D no change is seen as compared to control. G and H show sequestration of Nmd3 in the nucleus because of export defects as compared to the control (E and F).

Figure 7: *RLP24*-myc expression in *nup159*^{ts} causes export defects as observed with Rpl25-GFP and Nmd3-GFP



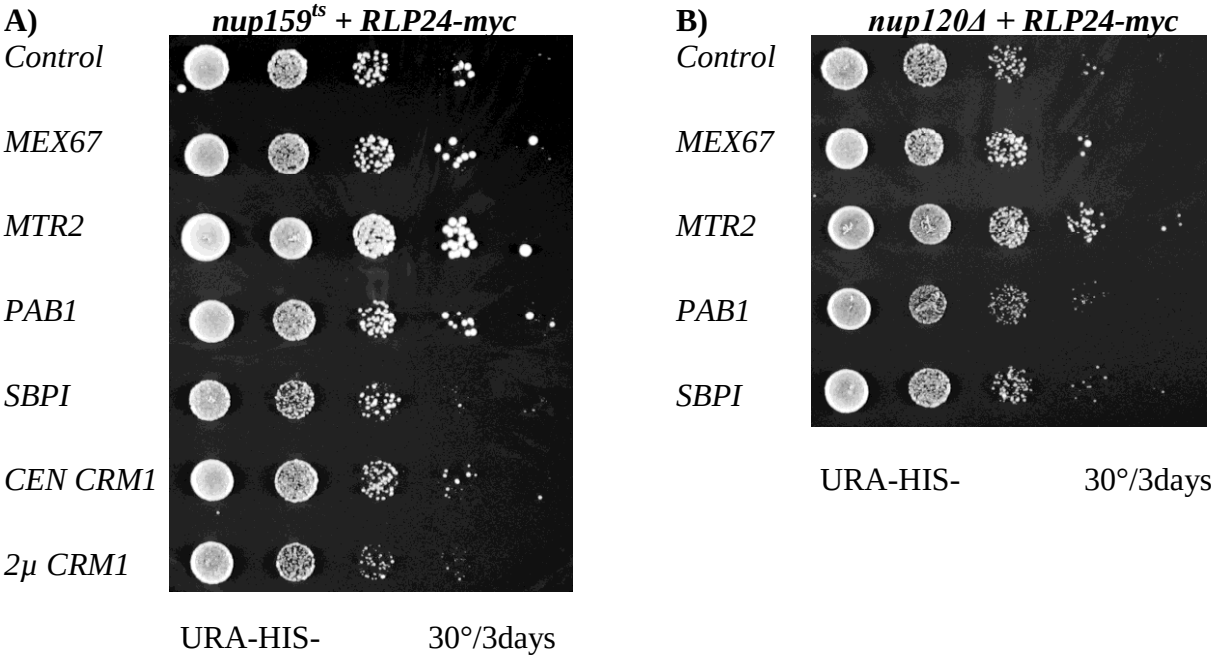
Panels A, B, E, and F show normal localization for Rpl25-GFP and Nmd3-GFP in a *nup159*^{ts} strain. In C and D nuclear preference is seen as compared to control, but the signal is still present in the cytoplasm. G and H show extreme sequestration of Nmd3 in the nucleus because of export defects as compared to the control (E and F).

Only the *nup120Δ* and the *nup159*^{ts} mutants showed enhanced nuclear localization and sequestration of Nmd3-GFP, suggesting these mutants have ribosomal export defects as a result of *RLP24*-myc expression. The other strains tested did not exhibit export defects by this method.

Potential High Copy Suppressor Tests

Because of the growth studies of the *RLP24*-myc expressing mutant strains, the search for a gene that could suppress the growth defect was initiated. Differences in high copy suppressors could possibly differentiate *RLP24*-myc D/N effects from those of *NMD3* or identify the target that Rlp24 interacts with. A panel of potential genes was first tested. This group was comprised of genes known to be involved in export or implicated as interactors: *CRM1*, *MTR2*, *MEX67*, *SBP1*, and *PAB1*. These genes were transformed into two strains from the expression study above selected for their strong D/N phenotypes, *nup120Δ+RLP24*-myc and *nup159^{ts}+RLP24*-myc. The *CRM1* transformations into the *nup120Δ* strain render the cells extremely sick and slow growing, so they could not be grown to sufficient density for growth tests. In both strains, *MEX67* and *MTR2* showed slight suppression of the dominant negative phenotype (**Figure 8**).

Figure 8: *MTR2* and *MEX67* show slight suppression of the D/N phenotype



Test for potential high copy of suppressors of D/N *RLP24*-myc phenotype in *nup159^{ts}* and *nup120Δ* strains. Both panels (**A** and **B**) show slight suppression with *MTR2*and *MEX67* but otherwise no phenotypic change.

High Copy Suppression Screen for* RLP24*-myc Growth Defect

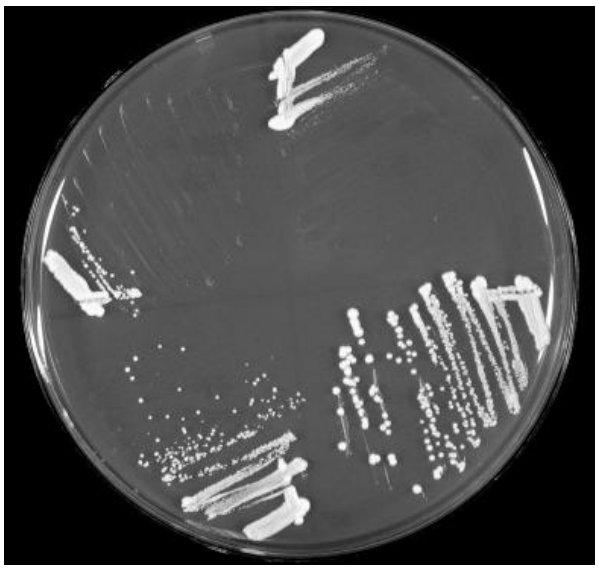
Following the test for high copy suppression by a potential panel of genes, a wide scale screen was done to identify high copy suppressors. One strain was selected for this screen from the two tested above: *nup159^{ts}+RLP24*-myc. A high copy URA3 library was transformed into the *RLP24*-myc containing *nup159^{ts}* strain and colonies were selected based on rescue of the growth defect. Colony selection was based on colony size greater or comparable to the control (*nup159^{ts}+ RLP24*-myc + empty vector). These clones were then transferred to HIS- 5FOA plates to select against the suppression vector and maintain the *RLP24*-myc vector. If suppression is due to the presence of the URA3 library plasmid, the colony size should then be decreased and appear like the *nup159^{ts}+RLP24*-myc mutant as no suppression is occurring.

From the plates, 102 colonies were identified with higher or equal growth compared to the control on URA-HIS-. Of those, 28 showed extremely high growth on URA-HIS- in combination with severely inhibited growth on HIS-5FOA. DNA from these clones was isolated and transformed into *E.coli*. After isolation from the bacteria, the plasmids were diagnostically digested with restriction endonucleases to confirm the plasmids did not contain *RLP24*-myc or wild-type *NUP159*. Plasmids not identified as either of those plasmids were transformed back into yeast to confirm suppression (Clones #2, 8, 9, 10, 11, 13, 14, 21, 28, 30, 31, 32, 36, 40, 43, 87, and 98). Only 7 clones confirmed suppression and were sequenced: #2, 8, 9, 14, 31, 40, and 87.

The presence of wild-type *NUP159* was confirmed in plasmid 31 as well as plasmid 80 which was sequenced in addition to the suppressor plasmids as a *NUP159* control. Three plasmids (2, 9, and 14) contained the *URA3* gene. The three remaining plasmids produced unique genes and were the only potential high copy suppressors identified. Plasmid 8 contained the sequence from chromosome 12 with open reading frames for *BRE2*, *PML1*, and *MEU1*.

Plasmid 87 contained the sequence from chromosome 15 with ORFs for *LIP5*, *MCA1*, *BFR1*, and *MRM1*. Plasmid 40 did not produce 5' sequencing results so the exact identity of the region is not confirmed, but the 3' sequencing identifies the same region as plasmid 87. This redundancy suggests strong high copy suppressor function. Of the ORFs contained in the insert, the most likely suppressor is a gene called *BFR1*. It is a component of mRNP complexes associated with polyribosomes implicated in secretion and nuclear segregation. **Figure 9** below shows the rescue of potential high copy suppressor 87 and its phenotype as compared to the *nup159*^{ts} mutant and *nup159*^{ts}+*RLP24*-myc mutant. I am currently subcloning the different genes from the suppressing plasmid #87 to identify which gene is responsible for suppression.

Figure 9: Plasmid 87 is a High Copy Suppressor of *RLP24*-myc Dominant Negative Phenotype



The streak plate above shows a comparison between the *nup159*^{ts} mutant, *nup159*^{ts} with *RLP24*-myc, and potential high copy suppressors 80 and 87. Plasmid 80 contains the sequence for wild-type *nup159* and serves as a control as well. Plasmid 87 restores growth to *nup159*^{ts} levels.

Yeast Two-Hybrid Interactions with Rlp24

The yeast Two-Hybrid system has traditionally been used as a way to find proteins that physically interact with a protein of interest, in this case Rlp24. The gene of interest is fused to a GAL4 binding domain sequence in a plasmid, which when expressed in cells produces a fusion protein. In this experiment, four fusion constructs were made for Rlp24: full-length, N-terminus deletion, C-terminus deletion, and a double N- and C-terminal-deletion. Rlp24 differs from its cytoplasmic counterpart Rpl24 in the C-terminus region, suggesting this region may mediate the role, if any, Rlp24 has in export. In addition, the effect of C-terminal tags on Rlp24 could be explained by their interfering with the interaction of another protein in this region of Rlp24. This region may interact with nucleoporins or recruit other export factors, mediating the export function of Rlp24.

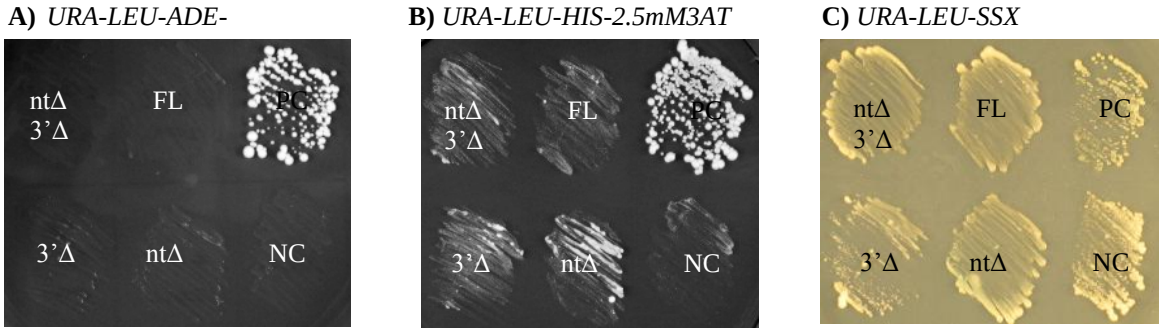
Potential interactors are fused to a GAL4 activation domain in the same manner as above. Plasmids containing these gene fusions are cotransformed into a strain containing a GAL4 promoter that controls transcription of reporter genes. If functional interaction occurs, then the target and prey proteins bind together, bringing the GAL4 binding domain and activation domain in close proximity. This allows them to activate transcription of the reporter genes controlled by the GAL4 promoter: histidine production, adenine production, and β-galactosidase production. These reporters can be identified by growth on selective media and by a colorimetric assay.

To ensure that the *RLP24*-GBD constructs did not themselves activate GAL4 transcription, a self-activation test was performed with cotransformation of each of the *RLP24* constructs and an empty activation domain. Transformants were first plated on URA-LEU- to select for the two vectors, then transferred to URA-LEU-ADE- and URA-LEU-HIS with differing concentrations of 3AT. As expected, no growth was seen on the ADE- plates. Because the histidine production is a less stringent reporter, the drug 3AT is added to minimize

background. A concentration of 2.5 mM 3AT was identified as sufficient to reduce background (false positive) growth (data not shown).

After demonstrating that self-activation did not occur, a test for activation with a potential interactor Drg1 was done. The *DRG1*-GAD plasmid was cotransformed with each of the *RLP24*-GBD constructs. Transformants were plated on the selective media. After 14 days of incubation, no growth was seen on the URA-LEU-ADE- plates while minimal growth was seen on URA-LEU-HIS- 2.5mM 3AT. Additionally, growth on URA-LEU- SSX did not produce any blue precipitate, indicating β-galactosidase was not expressed (**Figure 10**). The blue precipitate is produced in low levels in the positive control not visible in the picture. The media was then altered to produce more clear results for β-galactosidase production.

Figure 10: Drg1 shows no functional interaction with Rlp24 by Two-Hybrid Screen



Two-hybrid interactions between Rlp24 and Drg1. The patches are, clockwise from upper left, double deletion Rlp24, full-length Rlp24, positive control, negative control (empty vectors), N-terminus deletion Rlp24, and C-terminus deletion of Rlp24. (A) No growth is seen by experimental transformants on URA-LEU-ADE-. Slight growth is seen on URA-LEU-HIS-, however, there is no significant difference as compared to the negative control (B). Additionally, the level of background is much higher for this reporter. Finally, panel (C) shows no blue substrate is produced in the presence of XGAL.

Yeast Two-Hybrid Screen with Full-length Rlp24

The Two-Hybrid test was expanded into a genome wide screen, using libraries of GAD fusion plasmids with genomic regions of 500-3000 base pairs (James, Halladay et al. 1996).

Three libraries of these GAD fusion plasmids were constructed with the same inserts but with different frameshifts, ensuring that all inserts would be expressed in each of the three reading frames. These libraries are denoted C1, C2, and C3 based on the number of the nucleotide frameshift. The Two-Hybrid compatible strain (PJ69-4A) was transformed with *RLP24*-GBD and the each of the libraries using a large scale high efficiency transformation. Transformations were initially plated on URA-LEU-HIS- 2.5 mM 3AT and then patched to the selective media to test for the other reporters. From the C1 library, 4.7×10^6 transformants were screened, of which 70 were positive for histidine production. Of those, only one also showed growth on URA-LEU-ADE- and blue substrate production on URA-LEU- SSX (C1 #44). However, despite repeated attempts, the plasmid containing the GAD could not be isolated from this mutant. This suggests that perhaps the plasmid was not carrying a functional interactor but that the strain acquired a genomic mutation that resulted in expression of the reporters. Another possibility is that the plasmid had been integrated. In the C2 library screen, out of 1.3×10^6 transformants 30 colonies were initially isolated as His+, but only 1 produced all three reporters (C2 #1). Sequencing results from this plasmid identified the GAD fusion protein as Vps75. Finally, of the 1.1×10^6 C3 transformants, 47 colonies were isolated by histidine production but none tested positive for all three reporters.

Yeast Two-Hybrid Screen with RLP24 with N-terminus Deletion

To identify proteins that interacted specifically with the C-terminus of Rlp24, a Two-Hybrid screen was performed using only this region (ntΔ) as "bait." The *ntΔ RLP24*-GBD plasmid was cotransformed with the C1, C2, and C3 libraries as above. The C1 library transformation yielded 2.01×10^6 transformants of which 62 colonies grew on URA-LEU-HIS-2.5mM 3AT. Only one transformant produced all three reporters, C1 #62. After sequencing, this clone was determined to contain the C-terminus region of *NOG1*. Nog1 has been cited in the

literature as a functional interactor with Rlp24, insuring a real positive result and a control for confidently asserting the success and validity of this screen. The C2 library originally produced 79 colonies from 1.91 x 10^6 transformants, but none tested positive for all reporters. The C3 library resulted in 81 colonies from 1.66 x 10^6 transformants where two exhibited all reporters: C3 #6 and C3 #7. Sequencing was not successful for clone #6, but from diagnostic enzyme digestion appears with identical banding patterns to C3 #7. It is possible that these clones were cross contaminated or are two isolates of the same clone. C3 #7 sequencing showed regions of the genes *NDL1* and *GUP1*, both of which reside on different chromosomes. They do share domains in common, but neither seems to be spatially or functionally associated with export and are likely not probative as a potential interactors.

Discussion and Conclusions

In the work presented in this thesis, I first showed that an epitope tagged allele of *RLP24*, a gene encoding an essential ribosome biogenesis factor, displayed a genetic interaction profile similar to the 60S biogenesis export adapter Nmd3. This implicates Rlp24 in nuclear export of the 60S ribosomal subunit. I then used this observation in a dosage suppression screen for genes that functionally interact with Rlp24 in export. Finally, I used a Two-Hybrid genetic screen to identify proteins that physically interact with Rlp24. From this study, several new and unstudied interactions between Rlp24 and other genes and proteins have been elucidated. Though these results do not create a comprehensive model of Rlp24's role in the export of the large ribosomal subunit, they provide a starting point for further research. The significance of the interactions that I identified is not yet fully determined, but their incorporation into new and known models will be incredibly useful in the future.

From the initial expression study of *RLP24*-myc, several interesting conclusions were made. First, *RLP24*-myc expression caused a dominant negative phenotype similar to that seen with over-expression by *NMD3*. It is interesting that the same NPC deficient strains would exhibit this phenotype with both *RLP24*-myc and *NMD3*. This may indicate that like *NMD3*, *RLP24*-myc is preventing the binding of export receptors to the ribosome, therefore inhibiting efficient export. It can also be postulated that it displaces endogenous *RLP24* at a high enough level to prevent any necessary interactions. Surprisingly, the over-expression of other export receptors did not rescue the growth phenotype. It is assumed that such over-expression may overcome the block of export by up-regulating a tandem pathway, providing a missing downstream component, or providing an excess of the export receptor that is blocked by *RLP24*-myc. Only increased *MTR2* and *MEX67* showed slight suppression, but the effect was modest and we do not know if it reflected a real defect in recruitment of Mtr2 to the ribosome.

Additional experiments are needed to assess the abundance of Mtr2 on 60S subunits in the presence of Rlp24-myc.

Genetic interactions identified between Rlp24 and the Nuclear Pore Complex were specific to two major subcomplexes inside the NPC. The first contains nup120, nup133, and nup84 and is part of the membrane-coating complex on the interior of the pore (Alber, Dokudovskaya et al. 2007). This creates a kind of scaffold for the structural core for the NPC. The second subcomplex contains nup49, nup159, nup 116, and nsp1 (Alber, Dokudovskaya et al. 2007). These proteins contain the aforementioned FG repeats which occlude the center of pore and block the passage of molecules (Alber, Dokudovskaya et al. 2007). It is possible, therefore, that Rlp24 acts as an additional partitioning factor to allow passage of the large ribosomal subunit.

These nucleoporins were tested because of previous implication as being involved with known export receptors. Specifically, both of these subcomplexes have been shown to interact genetically and functionally with the Mtr2-Mex67 export complex (Strasser, Bassler et al. 2000). The authors assert that interactions of Mtr2-Mex67 with the nup84 containing complex, which may be dynamic *in vivo*, may help to localize the export receptor to the NPC. Interactions with the FG containing nups may help to dissociate the interactions and so-called "hydrophobic sieve" on the interior of the pore, allowing passage of large molecules (Strasser, Bassler et al. 2000). The fact that *RLP24*-myc not only shares the same phenotypic effect on nup mutants as other export receptors but that the spectrum of these interactions exactly parallels those of established receptors supports the hypothesis that Rlp24 is indeed involved in the export of the 60s subunit. Rlp24-myc appears to interfere with the function of an export receptor by possibly impairing an interaction between the 60S subunit and the NPC.

The high copy suppressor screen was used as a way to tease apart the mechanisms of the dominant negative effect seen in the nup mutants and thus export functions of Rlp24 and Nmd3. As mentioned previously, potential high copy suppressors such as Crm1, Mtr2, and Mex67 did not show significant suppression of the phenotype. When this was expanded into a genome wide screen, only one suppressor was isolated. This high copy suppressor (#87) contained open reading frames for *LIP5*, *MRM1*, *MCA1*, and *BFR1*, all contained on a region of chromosome 15. Putative functions were investigated through data compiled in the *Saccharomyces* Genome Database. *LIP5* is involved in the production of lipoic acid while MetaCAspase1 (*MCA1*) is thought to be related to caspases involved in regulating apoptosis in cells. Mitochondrial rRNA Methyltransferase (*MRM1*) is responsible for an essential methylation on mitochondrial rRNA. Finally, BreFeldin Resistant 1 (*BFR1*) is a component of mRNP complexes associated with polyribosomes. Out of all of the known functions of the genes contained in the high copy suppressor, the most intriguing possibility of interaction with Rlp24 is Bfr1. Localization studies show that Bfr1-GFP is cytoplasmic with preference to the nuclear membrane. Membrane association was confirmed with differential centrifugation as the majority of Bfr1 was found in the membrane pellet (Lang, Li et al. 2001). This shows that Bfr1 is physically located at the exact position to be involved in export. Perhaps it too interacts with the Nuclear Pore Complex on the cytoplasmic face, coming into contact with the ribosome as it exits the channel.

Further characterization of this high copy suppressor is a priority in follow-up experiments. By subcloning the different genes of the known suppressor plasmid, we can isolate which gene is actually responsible for the suppression phenotype. Further investigations can then be done to determine what place it has in export, if any. It is also possible that the suppressing gene acts by a mechanism other than directly suppressing an export defect. For example, the high copy suppressor may attenuate expression of *RLP24*-myc. The dominant

negative phenotype would be correspondingly decreased. This experiment would help address this issue as well as identify the component of the plasmid responsible for suppression.

Using the Yeast Two-Hybrid technique, I was able to identify proteins that potentially physically interacted with Rlp24. For each of the genomic libraries, confidence was in excess of 95% that the entire genome had been covered in the transformations. However, when looking at the full-length Rlp24, only one physical interaction was identified: Vps75. It is difficult to know *a priori* if the physical interactions identified by Two-Hybrid are biologically relevant. However, the Vps75 protein was previously identified by mass spectrometry as a member of the Rlp24 preribosome complex (Saveanu, Namane et al. 2003). We are therefore encouraged that this interaction is indeed real, but it is a new and uncharacterized member of the preribosome complex. Initial characterization of Vps75 revealed a role in vacuolar protein sorting, assisting newly made proteins in their passage from the Golgi to vacuoles (Bonangelino, Chavez et al. 2002). Further characterization identified a role as a NAP histone chaperone with a possible function in chromosome assembly (Selth and Svejstrup 2007). This protein seems to have a wide array of functions in many areas of the cell, especially with the addition of its presence in the preribosome complex. Perhaps it retains some of its chaperone functionality in the export of the ribosome, a process that requires further research and characterization. Alternatively, it could coordinate disparate cellular processes.

Because Rpl24 and Rlp24 differ most significantly in their C-terminal ends, this suggests a role of the C-terminus of Rlp24 in export function. Additionally, the presence of the myc-epitope tag on the C-terminus of Rlp24 caused impaired function in cells. In conjunction, these pieces of evidence may indicate that the C-terminus of the protein is essential for function. To test this further, we used the N-terminal deletion construct in the Two-Hybrid screen to see if the smaller and more functional region would draw out more interactions specifically related to

export. Indeed, one resulting interaction was Nog1. Nog1 is known to be tightly associated with both Nop7p and Rlp24 in a complex and is involved in both early and late ribosome maturation (Honma, Kitamura et al. 2006). This result was important for two reasons. First, retrieving a well characterized interaction between Rlp24 and another protein serves as a positive control for the screen and ensures that the screen is working properly. This gives confidence that other interactions with the Rlp24-GBD construct are valid. Secondly, the region of Nog1 that was expressed in the GAD vector was only the c-terminus, showing that both c-terminal ends of Rlp24 and Nog1 may physically associate and could be integral to their functionality. Alternatively, it could be necessary for proper regulation.

During this study, an article was published suggesting the protein Drg1 was involved in recycling 60S preribosomal particles (Pertschy, Saveanu et al. 2007). Drg1 copurifies in small amounts with both Rlp24 and Nog1, and when it is depleted, pre-60S factors including Rlp24 accumulate in the cytoplasm (Pertschy, Saveanu et al. 2007). This suggests both a physical and functional interaction between Rlp24 and Drg1. The Two-Hybrid system was used to investigate this physical interaction, and a *DRG1*-GAD fusion plasmid was coexpressed with the *RLP24*-GBD constructs of all lengths. The reporters were weakly expressed if at all present, therefore not allowing us to confirm the interaction by Two-Hybrid. This type of result shows that perhaps the screen may have not been sensitive enough to detect all putative interactions. Of course, some genes in the library constructs will be truncated, and some proteins will therefore not be represented. Though the Two-Hybrid screen is an extremely informative tool, it must be supplemented by additional studies and tests.

This series of experiments has provided significant evidence to implicate Rlp24 in the export of the large ribosomal subunit from the nucleus. Building on the initial studies showing shuttling of Rlp24 with the ribosome during export, we have genetic established interactions with

several of the proteins comprising the Nuclear Pore Complex. These same nucleoporins have been identified as interacting with other known export receptors, specifically Mtr2-Mex67. Furthermore, interactions between Rlp24 and Nog1 as well as Vps75 have been supported by Two-Hybrid analysis. Though the Nog1 interaction has been investigated, Vps75 offers a novel interaction in which its function in this system is entirely unknown. Finally, a putative interaction between Bfr1 and Rlp24 was identified. Though its localization and previous functional characterizations suggest it is involved in translation in the cytoplasm, this interaction needs to be confirmed by further analysis, followed by investigation in regards to its role with Rlp24 or in export. This body of work elucidates a number of interactions, known and novel, many of which are avenues for continuing research. This lays the foundation for investigation into Rlp24 as a protein involved not only in ribosome biogenesis but in export of the large ribosomal subunit from the nucleus.

Materials and Methods

Yeast Strains and Media

Strains were grown at 30° unless otherwise indicated (for temperature sensitive strains) in yeast extract peptone (YP) media or media lacking certain amino acids (dropout). Dropout media is used to select for strains with plasmids containing the ability to supplement the missing amino acids (Standard Lab Protocols from the lab of A. Johnson). Some of the yeast strains used in this study contain deletions or temperature sensitive mutations in several nucleoporins or export receptors, including *nup120, nup84, nup59*^{ts}, *nup133, nup40, nup116*^{ts}, *nup49*^{ts}, *nup1, nup42, nup100, nup2, nup53, nup60, nsp1*^{ts}, and *ARX1*. A complete listing of strains and genotypes is included in **Table 1** in the appendix.

Expression of *RLP24* in Nup Deficient Strains

A 13-myc epitope tag fused to *RLP24* in a plasmid (pAJ 1891) was transformed into nup mutant strains AJY 1608, 1930, 1931, 1987, 1991, 1992, 1993, 1995, 1998, 2470, 2471, 2478, 2479, 2480, and 2481. Additionally, a control *URA3* plasmid (pAJ 100), an unaltered *RLP24* (pAJ1890), and a high copy expression vector of *NMD3* (pAJ 411) were transformed into the same strains as controls to compare the growth rate.

Resulting transformants were inoculated in 2 ml cultures overnight and diluted to an O.D. of 1. 5 microliters of 10x serial dilutions (10^0 to 10^{-4}) were plated on URA- in triplicate with one plate each incubated at room temperature (25°), 30°, and 33°. Growth rates were observed beginning at 3 days of incubation and photo documented.

Additionally, two strains were used to examine the effect of 2μ expression of *RLP24* as compared to *RLP24*-myc and *NMD3* as well as the effect of a different epitope tag, HA, on the

RLP24-myc D/N effect. AJY 1930 and 1931 were transformed with empty vector (pAJ100), 2µ *NMD3* (pAJ411), CEN *NMD3* (pAJ 409), CEN *RLP24* (pAJ 1890), 2µ *RLP24* (pAJ 2053), CEN *RLP24*-myc (pAJ 1891), and CEN *RLP24*-HA (pAJ 898). Serial dilutions of transformants were plated as above and growth rates examined and photodocumented beginning at 3 days of growth.

Fluorescence Microscopy of Export Defects

To determine whether *RLP24*-myc mutants blocked export, mutant strains were cotransformed with either Rpl25-GFP (pAJ 908) or Nmd3-GFP (pAJ 755) fusion proteins and *RLP24*-myc (pAJ1858) or *RLP24*-HA (pAJ 1139). Transformed strains (AJY 1930, 1931, 1991, 1992, 1608, and 1988) were inoculated in 2 ml overnight culture at 30° and then diluted about 10 fold in the morning. Cells were allowed to grow for approximately 6 hours and then spun down. Cells were resuspended in about 100 µl of supernatant and 2 µl were added to a glass slide and pressed with a coverslip. Cells were viewed at 1000x with brightfield and also illuminated with UV light to view GFP expression. Photos were taken at automatic exposure for brightfield and at 2 seconds exposure for GFP expression.

High Copy Suppressor Screen

Potential High Copy Suppressor Tests

Of the nup mutants with dominant negative *RLP24*-myc expression examined, two were selected for their strong phenotypes: *nup120Δ* and *nup159*^{ts}. A panel of genes related to export was over-expressed in these strains with *RLP24*-myc in order to see if the gene could suppress the growth defect. The genes used were CEN *CRM1*, 2µ *CRM1*, *MEX67*, *MTR2*, *PAB1*, and *SBP1* (pAJ 1853, 2055, 1872, 1877, 1880, and 1881 respectively). *RLP24*-myc was co-

transformed with each of the plasmids, and transformations were plated on URA-HIS- plates until colonies were seen. Transformants were inoculated in 2 ml overnight cultures and examined with a serial dilution growth test.

Transformation of High Copy Library

One of the strains used above was selected for a high copy suppressor screen to search for a gene to rescue the growth defect: *nup159*^{ts} + *RLP24*-myc (AJY1988 + pAJ 1858). The library used for the screen contained genomic inserts of 6-8kb segments in a *URA3* 2µ plasmid (pRS202, Sikorski and Hieter 1989). According to analysis, 8/9 transformants show the insert, so approximately 1.4 to 1.8 x 10^3 transformant colonies would be necessary to cover the yeast genome once. However, at least 3-4 times that number was achieved to ensure the entire genome was represented.

Two large scale transformations were done for a total of 49 plates as well as 5 control plates to use for efficiency counts as well as colony size. High Efficiency Yeast Transformation protocols were used. Strain AJY 1988+pAJ 1858 was grown to a density of 2x10^7 cells/ml in 50ml culture. Cells were resuspended in PEG/LiAc and approximately 10^8 used per transformation. 100µg of ssDNA and approximately 1.5 µg of plasmid DNA were added. Transformations were incubated at 30° for 30 minutes and heat shocked at42° for 15 minutes. Each transformation was divided and spread over 5 URA-HIS- culture plates and kept at 30° until colonies were seen.

Selection of HC Suppressors

Colonies were selected that were either larger or the same size as control and restreaked onto URA-HIS- plates. After 2 days of growth, transformants were restreaked again to HIS-

plates to prepare for selection against the library HC suppressor on HIS-5FOA. With the suppressor plasmid removed, growth should resemble the original strong dominant negative phenotype. Those colonies showing both increased growth with the HC suppressor on URA- HIS- and decreased growth without it on HIS- 5FOA as compared to control were cultured, and the suppressor plasmid removed by Yeast DNA Miniprep. After the HC suppressor plasmids were isolated, they were transformed into *E. coli* for replication. *E. coli* Boiling Lysis Miniprep was used to isolate plasmid DNA.

Diagnostic Enzyme Digestion

To confirm that the HC suppressor plasmids isolated were not the *RLP24*-myc vectors, digestion of each plasmid was done with different combinations of restriction endonucleases (New England Biolabs). DNA was digested in three separate reactions with XbaI/BamHI, HindIII/BamHI, and HindIII in appropriate NEB buffer and incubated at 37° for 2 hours. Products were run on a 1% agarose gel at 100V for 30 minutes. Those with unique band patterns as compared to the control *RLP24*-myc (pAJ 1858) digestion were sent to sequence. Sequencing was done with the forward T7 primer 5' TAATACGACTCACTATAGGG 3' and the reverse M13 primer 5'CAGGAAACAGCTATGACC 3'.

Yeast Two-Hybrid Screen

PCR of RLP24 *and Construction of* RLP24-*GBD Plasmids*

RLP24 was amplified from genomic DNA using AJO 1011 5' CGTGGATCCATGAGAATTTATCAATGC 3', AJO 1012 5' CGTGGATCCACTTTTG CCCAAAGAAGA 3', AJO 1013 5' GCAGTCGACTAAAAAGCAATTTTCTTTGTA 3', and AJO 1014 5' GCAGTCGACTAA GCTCTTTCTTGTTCTTTAGC 3'. For full-length segment,

AJO 1011 and AJO 1013 were used. AJO 1012 and AJO 1013 resulted in a 208 nt deletion at the N terminus of *RLP24* and AJO 1011 and AJO 1014 resulted in a 3' deletion of *RLP24* from nt 400. AJO 1012 and AJO 1014 resulted in a double deletion. PCR products and pAJ 866 (pGBDU – Gal4 Binding Domain) were digested with BamHI and SalI to create compatible ends for ligation. Each PCR product was ligated into pAJ 866, transformed into *E.coli*, isolated, and confirmed by sequencing (catalogued as pAJ 2009-2012).

Construction of* DRG1-*GAD Plasmid

Gene *DRG1* was amplified from genomic DNA using primers AJO 1093 5' CTGCCC GGGATGGCTCCTAAATCTAGTTC 3' and AJO 1094 5' CAGGGATCCTTACGAAGAT GAACCGCTTC 3'. PCR products and pAJ 553 (pGADU – Gal4 Activation Domain) were digested by KpnI and BamHI to create compatible ends. The PCR product was ligated into pAJ 553, transformed into *E.coli*, isolated, and confirmed by sequencing (catalogued as pAJ 2014).

Two-Hybrid Screen

To confirm non-activation by the binding domain alone, each *RLP24*-GBD construct (pAJ 2009-2012) was cotransformed with an empty activation domain plasmid (pAJ 553) into PJ69-4A. Transformants were plated on selective media for the GAL activated promoters: URA-LEU-ADE-, URA-LEU-HIS-. The drug 3AT was added at different concentrations in the URA-LEU-HIS- dropout media to suppress background growth, and a level of 2.5mM 3AT was established for the experimental protocol.

To check a probative functional interaction, each of the *RLP24*-GBD constructs was cotransformed with the *DRG1*-GAD plasmid (pAJ 2014) into the Two-Hybrid competent strain PJ69-4A. Transformants were plated on URA-LEU- to select for the two plasmids. Then,

transformants were transferred to selective media for Two-Hybrid interaction: URA-LEU-ADE-, URA-LEU-HIS- 2.5mM 3AT, and URA-LEU- SSX. Growth in the absence of adenine or histidine suggests or production of a blue substrate on SSX caused by cleavage of XGAL with GAL expressed β-galactosidase indicates Two-Hybrid interaction.

For the large scale screen, full-length *RLP24*-GBD (pAJ 2009) was transformed into strain PJ69-4A. A High Efficiency Transformation was performed as above with the resulting strain and the C1 pGAD library. Transformants were plated on 49 URA-LEU-HIS- 2.5 mM 3AT plates. One fifth of one transformation was plated in a dilution series on URA-LEU- to count colonies for a measure of efficiency. The confidence level in finding a desired clone was approximately 95% according to colony counts. A second large scale transformation was done as above with the C1 library but initially plated on URA-LEU-ADE-. Confidence for this transformation was 99.9%. Transformations for C2 and C3 pGAD libraries followed exactly as above and were plated on URA-LEU-HIS- 2.5 mM 3AT. Confidence levels for both libraries were at 95%. All colonies were streaked onto URA-LEU-ADE- and URA-LEU- SSX to confirm GAL promoter expression.

Following the full-length RLP24 screen, the N-terminusΔ *RLP24*-GBD construct (pAJ 2012) was used in an additional Two-Hybrid screen. Transformations of the C1, C2, and C3 libraries were done as above and plated initially on URA-LEU-HIS- 2.5 mM 3AT. Confidence levels for each were 99%. Colonies were checked for all reporters as above.

Any clones expressing all three reporters were plated on LEU-5FOA to select against the *RLP24*-GBD plasmid. Plasmid DNA was then isolated and transformed into *E. coli.* Plasmid DNA was isolated again and diagnostically digested to ensure the identity of the GAD clone. DNA was purified by Sigma Miniprep Kit for positive clones and sent to sequence with primers AJO 188 5’ TTCGATGATGAAGATACC 3’ and AJO 189 5’ TGAAGTGAACTTGCGGGG 3’.

Appendix

Table 1: Strains used in this study

Strain	***Genotype***	***Source***
PJ69-4A	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ	(James, Halladay et al. 1996)
AJY 1608	*MATα his3 leu2 ura3 nup120Δ::KanMX4*	(Hung, Lo et al. 2008)
AJY 1901	*MATa his3Δ1 leu2Δ0 ura3Δ0 ARX1Δ::KanMX4*	(Hung and Johnson 2006)
AJY 1930	*MATa his3Δ1 leu2Δ0 ura3Δ0 nup84Δ::KanMX4*	Open Biosystems
AJY 1931	*MATa his3Δ1 leu2Δ0 ura3Δ0 nup133Δ::KanMX4*	Open Biosystems
AJY 1942	*MATa BYU741 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
AJY 1987	*MATα gle2-1 (nup40) ade2-1 ura3-1 his3-11,15 leu2-3, 112 trp1-1*	(Stage-Zimmermann, Schmidt et al. 2000)
AJY 1991	*MATa nup116::HIS3 ts trp1 leu2 ura3 his3*	(Stage-Zimmermann, Schmidt et al. 2000)
AJY 1992	*MATα nup49-313ts ade3 ade3 his3 leu2 ura3 nup49::TRP1*	(Stage-Zimmermann, Schmidt et al. 2000)
AJY 1993	*MATα nup1-2::LEU2 his3Δ200 trp1-1 ura3-5 leu2-3,112*	(Stage-Zimmermann, Schmidt et al. 2000)
AJY 1995	*MATa nic96::HIS3 ade2 ADE3 ura3 trp1 leu2*	(Zabel, Doye et al. 1996)
AJY 1998	*MATα ng12Δ*	
AJY 2470	*MATa his3Δ1 leu2Δ0 ura3Δ0 nup42Δ::KanMX4*	Open Biosystems
AJY 2471	*MATa his3Δ1 leu2Δ0 ura3Δ0 nup100Δ::KanMX4*	Open Biosystems
AJY 2478	*MATa his3Δ1 leu2Δ0 ura3Δ0 nup59Δ::KanMX4*	Open Biosystems
AJY 2479	*MATa his3Δ1 leu2Δ0 ura3Δ0 nup2Δ::KanMX4*	Open Biosystems
AJY 2480	*MATa his3Δ1 leu2Δ0 ura3Δ0 nup53Δ::KanMX4*	Open Biosystems
AJY 2481	*MATa his3Δ1 leu2Δ0 ura3Δ0 nup60Δ::KanMX4*	Open Biosystems
VPS75Δ	*MATa his3Δ1 leu2Δ0 ura3Δ0 VPS75Δ::KanMX4*	Open Biosystems

Table 2: Oligonucleotides used for PCR and sequencing

Oligonucleotides	***Sequence***	***Target***
AJO 188	TTCGATGATGAAGATACC	sequencing GAD 5'
AJO 189	TGAAGTGAACTTGCGGGG	sequencing GAD 3'
AJO 1011	CGTGGATCCATGAGAATTTATCAATGC	RLP24 forward from ATG w/BamHI site
AJO 1012	CGTGGATCCACTTTTGCCCAAAGAAGA	RLP24 forward from nt208 w/BamHI site
AJO 1013	GCAGTCGACTAAAAAGCAATTTTCTTTGTA	RLP24 3' end with SalI anti
AJO 1014	GCAGTCGACTAAGCTCTTTCTTGTTCTTTAGC	RLP24 3'Δ w/SalI anti
AJO 1091	CTGGGTACCAGTGGGCCCGTGGTTTATCA	DRG1 5' upstream of start w/KpnI
AJO 1092	CAGGGATCCGAGTACTGCAATACACTTGG	DRG1 3' downstream of stop w/BamHI
AJO 1093	CTGCCCGGGATGGCTCCTAAATCTAGTTC	DRG1 5' from ATG w/XmaI
AJO 1094	CAGGGATCCTTACGAAGATGAACCGCTTC	DRG1 3' end w/BamHI
T7 primer	TAATACGACTCACTATAGGG	Forward sequencing of 2μ URA3 library
M13 Reverse primer	CAGGAAACAGCTATGACC	Reverse sequencing of 2μ URA3 library

Table 3: Plasmids used in this study

Plasmid	***Relevant Markers***	***Source***
pAJ 409	*URA3 CEN NMD3*	(Kallstrom, Hedges et al. 2003)
pAJ 410	*LEU2 2μ NMD3*	(Hedges, West et al. 2005)
pAJ 411	*URA3 2μ NMD3*	(Hofer, Bussiere et al. 2007)
pAJ 553	*LEU2 2μ pGAD C-1*	(James, Halladay et al. 1996)
pAJ 582	*LEU2 CEN NMD3-GFP*	(Hedges, West et al. 2005)
pAJ 755	*URA3 CEN NMD3-GFP*	(Hedges, West et al. 2005)
pAJ 866	*URA3 2μ PGBDU-C1*	(James, Halladay et al. 1996)
pAJ 898	*URA3 CEN RLP24-HA*	(West and Johnson, unpublished)
pAJ 908	*URA3 CEN RPL25-GFP*	(West, Hedges et al. 2005)
pAJ 1853	*HIS3 CEN CRM1*	(Lo and Johnson, unpublished)
pAJ 1858	*HIS3 CEN RLP24-myc*	(Lo and Johnson, unpublished)
pAJ 1872	*HIS3 2μ MEX67*	(Lo and Johnson, unpublished)
pAJ 1877	*HIS2 2μ MTR2*	(Lo and Johnson, unpublished)
pAJ 1880	*HIS2 2μ PAB1*	(Lo and Johnson, unpublished)
pAJ 1881	*HIS2 2μ SBP1*	(Lo and Johnson, unpublished)
pAJ 1890	*URA3 CEN RLP24*	(Lo and Johnson, unpublished)
pAJ 1891	*URA3 CEN RLP24-13myc*	(Lo and Johnson, unpublished)
pAJ 2001	*LEU2 CEN RLP24*	This Study
pAJ 2002	*LEU2 CEN RLP24-13myc*	This Study
pAJ 2003	*URA3 2μ nup159wt*	This Study
pAJ 2005	*URA3 2μ BRE2, PML1*	This Study
pAJ 2006	*URA3 2μ BFR1, MRM1, MCA1*	This Study
pAJ 2009	*URA3 2μ RLP24-GBD*	This Study
pAJ 2010	*URA3 2μ Δnt 3'Δ RLP24-GBD*	This Study
pAJ 2011	*URA3 2μ 3'Δ RLP24-GBD*	This Study
pAJ 2012	*URA3 2μ Δnt RLP24-GBD*	This Study
pAJ 2013	*URA3 2μ DRG1*	This Study
pAJ 2014	*LEU2 2μ DRG1-GAD*	This Study
pAJ 2015	*LEU2 2μ VPS75-GAD*	This Study
pAJ 2016	*LEU2 2μ c-termNOG1-GAD*	This Study
pAJ 2017	*LEU2 2μ Ndl1, GUP1-GAD*	This Study
pAJ 2018	*URA3 2μ MCA1*	This Study
pAJ 2019	*URA3 2μ MCA1, BFR1*	This Study
pAJ 2020	*URA3 2μ MCA1, BFR1, MRM1*	This Study
pAJ 2053	*URA3 2μ RLP24*	(Lo and Johnson, unpublished)
pAJ 2055	*HIS3 2μ CRM1*	(Lo and Johnson, unpublished)
pRS 413	*HIS3 CEN*	(Sikorski and Hieter 1989)
pRS 415	*LEU2 CEN*	(Sikorski and Hieter 1989)
pRS 416	*URA3 CEN*	(Sikorski and Hieter 1989)
pRS 423	*HIS3 2μ*	(Sikorski and Hieter 1989)
pRS 426	*URA3 2μ*	(Sikorski and Hieter 1989)

Table 4: Dominant Negative Phenotypes of RLP24-myc Expression in Nup Mutant Strains

AJY	Nup	Genotype	Role	Dominant Negative in nup mutants	
				CEN RLP24-myc	2μ NMD3
1608	Nup120	deletion	ribosome	Y (Strong)	Y
1930	Nup84	deletion	mRNA	Y (weak at 30 and 33)	Y
1931	Nup133	deletion	ribosome/mRNA	Y	Y
1991	Nup116	nup116ts (HIS3)	Ribosome/mRNA	Y	Y
1992	Nup49	nup49::Trp nup49-313ts(LEU2)	Ribosome	Y	Y
1990	Nup82	nup82::HIS3 pNup82-Δ108(URA3)	ribosome/mRNA	N	Y (weak at RT&30)
1988	Nup159	rat7-1 ts	Ribosome/mRNA	Y	Y (weak, only at 33)
1994	Nsp1	nsp1-10A ts(URA3)	Ribosome/mRNA	Y (weak @RT)	Y
1995	Nic96	nic96::HIS3 nic96-1(LEU2)	Ribosome	Y (weak at RT) N @30&33	Y (weak @ RT) N@30&33
1987	Nup40	gle2-1	Ribosome	N	N
1993	Nup1	nup1-2::LEU2 nup1-8 TRP1	Ribosome	N	N
2470	Nup42	Deletion	mRNA	N	N
2471	Nup100	Deletion	mRNA	N	N
2478	Nup59	Deletion	?	N	N
2479	Nup2	Deletion	?	N	N
2480	Nup53	Deletion	?	N	N
2481	Nup60	Deletion	?	N	N

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Biography

Kara Helmke was born in San Antonio, Texas on July 5, 1986. She attended Alamo Heights High School, graduating as class valedictorian in 2004. Kara enrolled in both the Plan II Honors Program and Dean's Scholars Honors Program at the University of Texas at Austin later that fall. While in school, she worked as an undergraduate researcher in two biology laboratories and as a peer tutor. In May 2008, she graduated with a B.A. in Plan II Liberal Arts and a B.S. in Biology Honors. She is currently pursuing a Ph.D. in Molecular and Cell Biology at the University of California Berkeley. Kara hopes to do research and teach at the university level upon conferral of her graduate degree.

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